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# *Indian Standard*

# METHOD FOR DETERMINATION OF PHOSPHATASE ACTIVITY IN MILK AND MILK PRODUCTS

## PART II REFERENCE METHOD

UDC 637·127·6 : 543·866

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# *Indian Standard*

# METHOD FOR DETERMINATION OF PHOSPHATASE ACTIVITY IN MILK AND MILK PRODUCTS

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# *Indian Standard*

# METHOD FOR DETERMINATION OF PHOSPHATASE ACTIVITY IN MILK AND MILK PRODUCTS

## PART II REFERENCE METHOD

## 0. FOREWORD

**0.1** This Indian Standard ( Part II ) was adopted by the Indian Standards Institution on 30 May 1977, after the draft finalized by the Dairy Products Sectional Committee had been approved by the Agricultural and Food Products Division Council.

**0.2** Phosphatase activity is determined to judge the efficiency of pasteurization of milk and milk products. To test whether the prescribed heat treatment was properly carried out, the treated milk or milk product is subjected to phosphatase test which helps to indicate the presence or absence of phosphatase enzyme. Phosphatase present in milk or milk products is destroyed by just about the same heat treatment necessary for the destruction of *Mycobacterium tuberculosis*, the most heat-resistant pathogen likely to be present in products. The method is, therefore, applied for the control of proper pasteurization.

**0.3** This standard is being issued in two parts. This part ( Part II ) covers the reference method for the determination of phosphatase activity which requires the use of a spectrophotometer and is usually applied in cases of dispute. Part I covers the routine method which is carried out with special discs and a comparator is used for day-to-day checks for the control of proper pasteurization of milk and milk products in a dairy.

**0.4** In the preparation of this standard, considerable assistance has been derived from ISO/R 3356 ' Milk and dried milk, buttermilk and buttermilk powder, whey and whey powder — Determination of phosphatase activity ( reference method ) ' issued by the International Organization for Standardization.

**0.5** In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS : 2-1960*.

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*Rules for rounding off numerical values ( *revised* ).

## 1. SCOPE

**1.1** This standard ( Part II ) specifies a reference method for the determination of the phosphatase activity in milk and milk powder; buttermilk and buttermilk powder; and whey and whey powder.

## 2. QUALITY OF REAGENTS

**2.1** Unless specified otherwise, pure chemicals shall be employed in tests and distilled water ( *see* IS : 1070 - 1977* ) shall be used when the use of water as a reagent is intended.

NOTE — ' Pure chemicals ' shall mean chemicals that do not contain impurities which affect the result of analysis.

## 3. TERMINOLOGY

**3.1** For the purposes of this standard, the phosphatase activity shall be the quantity of active alkaline phosphatase present in the product, expressed as the quantity of phenol, in micrograms, liberated under the specified conditions by one millilitre of the liquid product or, in the case of dried products, by one millilitre of the reconstituted sample.

## 4. PRINCIPLE

**4.1** Dilution of the liquid product or the reconstituted liquid product with a buffer at *p*H 10·6 and incubation at 37°C for one hour. Addition of disodium phenylphosphate liberating phenol by reaction with quinoneimide. Photometric measurement of the phenol liberated.

## 5. APPARATUS

**5.0** All glassware, stoppers and sampling tools shall be carefully cleaned. It is desirable to rinse them with freshly boiled distilled water. Certain types of plastics stoppers may cause phenolic contamination and their use shall, therefore, be excluded.

**5.1 Water-Bath** — maintained at 37 ± 1°C, thermostatically controlled.

**5.2 Spectrophotometer** — suitable for readings at a wavelength of 610 nm.

**5.3 Test-Tubes** — of size 16 or 18 mm × 150 mm, preferably graduated at 5 and 10 ml.

**5.4 Graduated Pipettes** — 1 and 10 ml.

**5.5 Filter Paper** — Whatman No. 42, S and S 597 or equivalent.

**5.6 Litmus Paper**

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*Specification for water for general laboratory use ( *second revision* ).

## 6. REAGENTS

### 6.1 Barium Borate-Hydroxide Buffer

**6.1.1** Dissolve 50·0 g of barium hydroxide [ $Ba(OH)_2.8H_2O$ ], free from carbonate, in water and make up the volume to 1 000 ml.

**6.1.2** Dissolve 22·0 g of boric acid ( $H_3BO_3$ ) in water and make up the volume to 1 000 ml.

**6.1.3** Warm 500 ml of each solution ( **6.1.1** and **6.1.2** ) to 50°C, mix the solutions. Stir and cool rapidly to about 20°C. Adjust the *p*H if necessary to 10·6 ± 0·1 by addition of solution prepared in accordance with **6.1.1** or **6.1.2** and filter.

**6.1.3.1** Store the solution in a tightly stoppered container. Dilute the solution before use with an equal volume of water.

**6.2 Colour Development Buffer** — Dissolve 6·0 g of sodium metaborate ( $NaBO_2$ ) or 12·6 g of $NaBO_2.\ 4H_2O$, and 20·0 g of sodium chloride ( $NaCl$ ) in water and make up the volume to 1 000 ml.

**6.3 Colour Dilution Buffer** — Dilute 10 ml of the colour development buffer ( **6.2** ) to 100 ml with water.

**6.4 Buffer Substrate** — Dissolve 0·5 g of disodium phenylphoshate ( $Na_2C_6H_5PO_4.\ 2H_2O$ ) in 4·5 ml of the colour development buffer ( **6.2** ). Add two drops of the BQC solution ( **6.6** ) and let stand at room temperature for 30 minutes. Extract the colour so formed with 2·5 ml of butanol-1 and let stand until the butanol-1 separates. Remove the butanol-1 and discard. Repeat this extraction if necessary. The solution may be stored in a refrigerator for a few days; develop the colour and re-extract before use. Prepare the buffer substrate immediately before use by diluting 1 ml of this solution to 100 ml with the barium borate-hydroxide buffer ( **6.1** ).

**6.5 Zinc-Copper Precipitant** — Dissolve 3·0 g of zinc sulphate ( $ZnSO_4.\ 7H_2O$ ) and 0·6 g of copper ( II ) sulphate ( $CuSO_4.\ 5H_2O$ ) in water and make up the volume to 100 ml.

**6.6 2,6-Dibromoquinonechloroimide Solution ( Gibb's Reagent)** — Dissolve 40 ± 1 mg of 2,6-dibromoquinonechloroimide ( BQC ) ( $O = C_6H_2Br_2 = NCl$ ) in 10 ml of 96 percent ( *v*/*v* ) ethanol.

**6.6.1** Store in a dark-coloured bottle in a refrigerator. Reject if discoloured or more than one month old.

**6.7 Copper Sulphate Solution** — Dissolve 0·05 g of copper ( II ) sulphate ( $CuSO_4.\ 5H_2O$ ) in water and make up the volume to 100 ml.

**6.8 Sodium Hydroxide Solution** — 0·5 N.

### 6.9 Standard Phenol Solutions

**6.9.1** Weigh 200 ± 2 mg of pure anhydrous phenol, transfer to a 100 ml volumetric flask, fill to the mark with water and mix.

**6.9.1.1** This stock solution remains stable for several months in a refrigerator.

**6.9.2** Dilute 10 ml of this stock solution to 100 ml with water and mix. One ml of this solution contains 200 $\mu$g of phenol.

## 7. SAMPLING

**7.1** While drawing and handling of the sample, same precautions as for bacteriological examination shall be observed.

## 8. PROCEDURE

**8.0** Avoid the influence of direct sunlight during the determination. Contamination with traces of saliva or perspiration may give false positive results and shall be avoided. In this respect special attention shall be devoted to the pipetting.

### 8.1 Preparation of the Test Sample

**8.1.1** *Milk, Buttermilk and Whey*

**8.1.1.1** Carry out the analysis preferably directly after sampling. Otherwise, keep the sample in a refrigerator, but not for more than 48 hours.

**8.1.1.2** Mix the sample carefully, if necessary with moderate heating. The temperature of mixing shall not exceed 35°C.

**8.1.2** *Milk Powder, Buttermilk Powder and Whey Powder* — Dissolve 10 g of the product in 90 ml of water. The temperature applied in dissolving shall not exceed 35°C.

**8.1.3** *Neutralization of Sour Products* — Add to a sour product sodium hydroxide solution ( **6.8** ) until a drop is neutral to the litmus paper.

**8.2 Test Portion** — Pipette into each of two test-tubes ( **5.3** ) 1 ml of the test sample ( **8.1** ), using one tube as a control or blank.

### 8.3 Determination

**8.3.1** Heat the blank for 2 minutes in boiling water, cover the tube and the beaker of boiling water with aluminium foil to ensure that the entire tube is heated. Cool to room temperature.

**8.3.2** From this step onwards, treat the blank and the test sample alike. Add 10 ml of the buffer substrate ( **6.4** ) and mix.

**8.3.3** Immediately incubate in the water-bath at 37°C for 60 minutes, mixing the contents occasionally.

**8.3.4** Heat in boiling water for 2 minutes in the same manner as under **8.3.1**. Cool to room temperature.

**8.3.5** Add one ml of the zinc-copper precipitant ( **6.5** ) into each tube and mix thoroughly.

**8.3.6** Filter through dry filter paper, discard the first drops; refilter if necessary until the filtrate runs clear and collect 5 ml in a test-tube.

**8.3.7** Add 5 ml of the colour development buffer ( **6.2** ).

**8.3.8** Add 0·1 ml of the BQC solution ( **6.6** ), mix, and allow the colour to develop for 30 minutes at room temperature.

**8.3.9** Measure the absorbance against the control or blank in the spectrophotometer at a wavelength of 610 nm.

**8.3.10** Repeat the determination with an appropriate dilution of the sample or the reconstituted sample if the absorbance as measured under **8.3.9** exceeds the optical density of the standard containing 20 µg of phenol per tube as measured under **8.4.4**.

**8.3.10.1** Prepare this dilution by mixing one volume of the test sample with an appropriate volume of a part of the same test sample heated carefully to boiling in order to inactivate the phosphatase.

### 8.4 Preparation of the Calibration Curve

**8.4.1** Provide a suitable range of diluted standard, starting from the standard phenol solution (**6.9.2**), containing 0 ( control or blank ), 2, 5, 10 and 20 µg of phenol per millilitre. Pipette respectively 1 ml of water and 1 ml of the four phenol standard solutions into each of five test-tubes.

**8.4.2** Add to each tube 1 ml of the copper sulphate solution ( **6.7** ), 5 ml of the colour dilution buffer ( **6.3** ), 3 ml of water and 0·1 ml of the solution ( **6.0** ), and mix.

**8.4.3** Allow the colour to develop for 30 minutes at room temperature.

**8.4.4** Measure the absorbance against the control or blank in the spectrophotometer at a wavelength of 610 nm.

**8.4.5** Prepare the calibration curve by plotting the optical densities against the quantities of phenol, in micrograms, indicated under **8.4.1**. The standard curve should be a straight line.

## 9. EXPRESSION OF RESULTS

### 9.1 Calculation

**9.1.1** Convert the absorbance as determined under **8.3.9** to micrograms of phenol by reference to the calibration curve.

**9.1.2** Calculate the phosphatase activity, expressed as micrograms of phenol by means of the following formula:

$$\text{Phosphatase activity} = 2{\cdot}4 \times A \times D$$

where

$A$ = quantity of phenol in micrograms obtained under **9.1.1**, and

$D$ = dilution factor of the dilution according to **8.3.10** ( in the case of no dilution, $D = 1$ ).

### 9.2 Repeatability of Results

**9.2.1** Repeatability of duplicate determinations ( results obtained simultaneously or in rapid succession by the same analyst ) shall not exceed 2 $\mu$g of phenol. If a dilution is applied according to **8.3.10**, this limit is referred to the results obtained on the diluted sample.

## 10. TEST REPORT

**10.1** The test report shall show the method used and the result obtained. It shall also mention any operating conditions not specified in this standard or regarded as optional, as well as any circumstances that may have influenced the result.

**10.2** The report shall include all details required for the complete identification of the sample.

( *Continued from page* 2 )

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